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Exploring the secrets of the three-dimensional architecture of phototrophic biofilms in caves

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Abstract:

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Caves with dim natural light, and lighted hypogean environments, have been found to host phototrophic microorganisms from various taxonomic groups. These microorganisms group themselves into assemblies known as communities or biofilms, which are associated with rock surfaces. In this work, the phototrophic biofilms that colonise speleothems, walls and floors in three tourist caves (Spain) were studied. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were used to study these organisms and acquire three-dimensional data on their biofilm structure. CLSM was used in a multi-channel mode whereby the different channels map individual biofilm components. Cyanobacteria, green microalgae, diatoms, mosses and lichens were found to be grouped as biofilms that differed according to the sampling sites. The biofilms were classified into six types regarding their environmental conditions. These types were defined by their constituent organisms, the thickness of their photosynthetic layers and their structure. Light-related stress is associated with lower biofilm thickness and species diversity, as is low humidity, and, in the case of artificially illuminated areas, the duration of light exposure.

Keywords: biofilms, caves, cyanobacteria, chlorophyta, Spain.

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INTRODUCTION

Biofilms are collectives of one or more species of microorganisms. They provide protection for growth, enabling microorganisms to survive hostile environments (Prakash et al., 2003) and are significant in sediment stabilization and construction (Golubic & Schneider, 2003). Biofilms comprise sessile microorganisms in different stages of growth; hence, they respond quickly to variable conditions (Costerton et al., 1987).

When microorganisms adhere to a surface, their immobilised cells grow, replicate and secrete extracellular polymeric substances (EPS) that engulf them in a gelatinous matrix (Brading et al., 1995). The development of complex, adhered or aggregated communities plays a key role in the survival and reproductive success of the microorganisms involved. Biofilms can provide refuge for species that face competition, predation or unfavourable environmental conditions (Korber et al., 1994).

Biofilms develop according to their environmental conditions and the physicochemical properties of their substrate (Walker & Pace, 2007). Research has shown that the coexistence of species in a biofilm depends on its capacity—and that of its competitors—to bind to its substrate (Stewart, 1997). Mixed biofilms are thicker and more stable to environmental stress than monospecific biofilms. This may be due to the production of a broader array of EPS by a greater variety of microorganisms (Kumar & Anand, 1998).

Natural biofilms can be highly organised, may encompass one or several species, and can form a single layer, a three-dimensional structure or even aggregates (Bryers, 1987, Bagge et al., 2001). The three-dimensional structure of a biofilm is related to its functions and to the survival of its constituent organisms and depends on environmental factors (Hall-Stoodley et al., 2004, Wimpenny et al., 2000). Biofilm structure can be dictated by numerous conditions, such as spatial and temporal differences in light and humidity, as well as surface and interface properties, nutrient availability and the composition of the microbial community (Davey & O'Toole, 2000). Biofilms differ in their architecture. Due to their

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depth, some biofilms are highly stratified (Zhang & Bishop, 1994).

To understand how certain microorganisms colonise their environments, these organisms not only need to be identified, but also understood at the level of community organisation (Albertano & Urzi, 1999; Hernández Mariné et al., 2001b, 2003; Roldán et al., 2004b). Likewise, the nature of the substrate can affect the binding pattern. For example, on hydrophilic surfaces, microorganisms tend to uniformly adhere as a monolayer, whereas on hydrophobic surfaces, they tend to adhere *en masse* (McEldowney & Fletcher, 1987), which is facilitated by their EPS.

Caves with dim natural light have been found to host phototrophic microorganisms from various taxonomic groups, including cyanobacteria, chlorophytes, bacillariophytes and lichens. Confined caves with a large volume of rock are usually characterised by homogeneous air temperatures and stable conditions. Humidity is usually high (Hernandez-Mariné et al., 1999, Roldán et al. 2004b). Limited photosynthetically active radiation (PAR) provides pressure for selection and is the principal determinant of whether a biofilm will tend towards autotrophy (i.e. algae and cyanobacteria) or heterotrophy (i.e. fungi and bacteria) as well as of the depth at which a biofilm will penetrate the cave interior. Communities of microorganisms organise themselves according to these gradients of light, whether natural or artificial. Nonetheless, neither the distribution of organisms within a rock, nor the balance between autotrophy and heterotrophy within a biofilm, is controlled exclusively by light. For example, the ratio of algae to bacteria can be influenced by the availability of nutrients or organic material (Ohki & Gantt, 1983).

Since the famous study of green sickness (Lefèvre et al., 1964), which affected the Paleolithic paintings in the Cave of Lascaux, France, the presence of photosynthetic microorganisms in the walls and paintings of caves and monuments has been reported. Artificially illuminated hypogean environments include tombs and Roman catacombs (Albertano et al., 2003). Cyanobacteria and bacillariophytes also have been reported in monuments such as the Parthenon in Athens (Anagnostidis et al., 1983), in construction materials in the cathedrals of Seville, Salamanca and Toledo (Ortega Calvo et al., 1991, 1993), and in Granada (Sánchez Castillo, 1981, 1983). Other studies have focused on cyanobacteria that affect stone monuments in tandem with lichens (Danin & Caneva, 1990). Additionally, microalgae have been observed growing on mural paintings (Grilli Caiola et al., 1987; Albertano et al., 1991) and on church frescoes (Pietrini & Ricci, 1993) in Italy. Many of the species found in these locations exhibit a tropical or Atlantic distribution and can be found in related environments such as land that lacks vegetation cover. The great diversity and broad distribution of photosynthetic organisms in these environments is reflected in the abundant literature on them (Hoffmann, 1989, 2002). Hypogean environments are typically equipped with artificial illumination, primarily for tourism. Depending on the intensity, quality and duration of the illumination, these areas can generate conditions which favour photoautotrophic growth, thereby enabling photosynthetic organisms, known as *Lampenflora* (Abdelahad, 1989; Smith & Olson, 2007) to extend into previously uncolonised areas (Hoffmann, 1989, 2002).

Hypogean environments have been studied on nearly every continent, namely, in Brazil (Sant'Anna et al., 1991); Israel (Friedmann, 1955, 1964; Vinogradova et al., 1998); Florida and the Cook Islands (Friedmann, 1979); the Bahamas (Davis & Rands, 1981); Hungary (Claus, 1964; Hajdu, 1966; Kol, 1966; Komáromy, 1977; Komáromy et al. 1985); France (Bourrelly & Dupuy, 1973; Leclerc et al., 1983); Germany (Dobat, 1977); Belgium (Garbacki et al., 1999); Italy (Abdelahad & Bazzichelli, 1988; Borzi, 1917; Skuja, 1970); Romania (Serbanescu & Decu, 1962); Croatia, Slovenia (Golubic, 1967); and Scotland (Carter, 1971). In Spain, caves and related environments have primarily been studied in Barcelona, Seville, Oviedo (Ariño et al., 1997; Hernández-Mariné et al., 2001b; Roldán et al., 2004b) and Murcia (Aboal et al. 1994; Asencio & Aboal, 1996, 2000).

The aforementioned locations host myriad cyanobacterial species, including *Myxosarcina*, in caves in Italy (Abdelahad, 1989); *Geitleria calcarea*, reported in caves (Friedmann, 1955) throughout France (Bourrelly & Dupuy, 1973; Leclerc et al., 1983), Italy (Abdelahad & Bazzichelli, 1988) and Spain (Gracia Alonso, 1974; Hernández-Mariné & Canals, 1994a); and *Scytonema julianum*, reported throughout Europe (Couté & Bury, 1988; Dobat, 1977; Garbacki et al., 1999; Hoffmann, 1989), including Spain (Ariño et al., 1997; Aboal et al. 1994; Hernández-Mariné & Canals, 1994a). Further study of these environments has led to observation of rare or even undocumented species such as *Herpyzonema pulverulentum* (Hernández-Mariné & Canals, 1994b), *Loriella* sp. (Hernández-Mariné et al., 1999) and *Symphyonema cavernicolum* (Asencio et al., 1996), filamentous cyanobacteria with calcified sheaths that have been reported in Spanish caves.

Another frequently observed group in caves is the Bacillariophyta. Whilst numerous species have been described on different continents, the most widely cited species are typically cosmopolitan and not very specific to the environments. Diverse species have been found in the United States (Saint-Clair & Rushforth, 1976), Scotland (Carter, 1971), Rome (Albertano et al., 1994) and Spain (Roldán et al., 2004b), including species from the genera *Diadesmis*, *Achnantes*, *Nitzschia*, *Cymbella* and *Orthoseira* (as *Melosira*).

Among chlorophytes in caves, the unicellular forms—the taxonomy of which is highly problematic—tend to dominate. These include *Bracteococcus minor* (Chlorophyta) (Lefèvre, 1974), the causative agent of green sickness in the Cave of Lascaux, France (Lefèvre et al., 1964). Chlorophyta species have been reported in other environments, including aeroterrestrial green microalgae (Crispim et al., 2003; Kumar et al., 2007; Tomaselli et al., 2000) or growing free living on tree

bark, soil and rock or as lichen photobionts (Ettl & Gärtner, 1995; Kumar & Kumar, 1999).

These organisms are named according to their position relative to the substrate (Golubic et al., 1981): those which colonise rock surfaces are known as *epiliths*, whereas those which colonise rock interiors are known as *endoliths*. The latter group includes *chasmoendoliths*, which colonise cracks in rocks.

The aims of the present study were to evaluate the biodiversity of photosynthetic microorganisms dwelling in the show artificially illuminated caves of Zuheros, Nerja and Collbatò (Spain) and to characterise the 3D structure of their respective biofilms, in order to provide information for improving control strategies.

MATERIALS AND METHODS

Source of the materials

We collected biofilm samples from three Spanish caves: Zuheros (Hernanz et al., 2006) and Nerja (Sanchidrián, 1986), which feature Palaeolithic rock paintings, and the show cave of Collbató. In all of these caves biofilm development was favoured by anthropogenic factors such as high CO_2 concentration and artificial illumination. Fragments of biofilms were collected together with small pieces of their support from rock substrata, speleothems, cave walls and floors.

The Cave of Bats is located at a height of 972 m a.s.l, close to the village of Zuheros (Córdoba, southern Spain). The continental Mediterranean climate of the area is characterised by a mean annual temperature of 14.9 ºC, ranging from –10 ºC in winter to over 40 ºC in summer, as well as by a high rate of rainfall, with maxima in November and February. The Cave of Bats has an opening at each end. These provide a high rate of natural ventilation, and relatively low air humidity compared to the other caves studied, and influences the internal temperature. Substrata consist of limestone rock that was partially covered by calcitic crusts and speleothems. Some areas were covered by bat droppings (Albertano et al., 2003). Six points in Zuheros cave were selected for study: Z1 to Z3 (speleothems or calcite with crust), which are located near the visitor entrance and receive both natural and artificial light; Z4 (white calcite), which have biofilms developed on dripping tracks and are illuminated artificially; Z6 is submitted only to artificial lighting for a short time during visits and Z7 and Z8 (calcite or speleothem), which are located in the exit hall and receive only natural light. PAR (photosynthetically active radiation) varies from 3.8 to 18 μmol m^{-2} s^{-1} at the entrance and from 0.05 to 5.0 μmol m^{-2} s^{-1} inside the cave (Albertano et al., 2003).

The Nerja Cave (Màlaga) is less than 1 km from the coast line. Its entrance is at 158 m a.s.l. The cave lies within dolomitic marble. Air temperature in the cave is ca. 19 ºC year round. Sampling site N5 is artificially illuminated during visits; sampling site N6 receives scarce natural light. The ambient humidity is between 84% and 99.9% at the sampling zones. Water from adjacent mountains enters the Cave, with maximum dripping in August.

The Salpetre Cave of Collbató (near Barcelona) is located at 500 m a.s.l. The representative sampling zone, Virgin Cave, is located at the bottom of the cave. The substrate comprises a flat, vertical calcite surface with clay impurities from decalcification. The only light that the Cave receives is artificial light during visits. It shows relative micro-environmental stability throughout the annual cycle: the mean value of the air temperature is 14.7 ºC, with an annual variation of 4.1 ºC; the mean value of the stone temperature is 15.5 ºC, with an annual variation of 1.2 ºC; and the ambient humidity is 99.9% at the sampling zone. Light is provided by incandescent lamps (Sylvania 80W, Holland).

Preparation and staining of material

We used an array of microscopy techniques to visualise the samples: scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) combined with lectin labelling.

SEM

Samples were processed with an acrolein-osmium fixation technique (Hernández-Mariné et al., 2001a) and viewed using a Hitachi S-2300 microscope. The biofilm was pre-fixed in acrolein vapour in a closed chamber with silica gel for 10 days. Post-fixation was carried out in osmium tetroxide vapour in the same chamber for an additional 5 days. Samples were double coated with carbon, using a rotary holder and sputtered gold. We used this technique for samples prone to collapse, as it affords adequate preservation of three-dimensional structures (Hernández-Mariné et al., 2001a). Small stones or filters may also be mounted on the stubs with double-sided adhesive tape and coated.

CLSM

The biofilms were examined using a confocal spectral Leica TCS SP2 (Leica Microsystems, Mannheim, Germany). All experiments employed a x10 (0.4 NA), a x40 (1.25 to 0.75 NA), and a x63 (1.32 NA) Plan Apochromat oil immersion objective. The 488 nm line of an Ar laser was used for imaging EPS (green channel, emission at 490 to 530 nm). EPS were labelled with the broad-spectrum, carbohydrate-recognizing lectin concanavalin-A (Molecular Probes, Inc., Eugene, OR, USA)—which binds to mannose and glucose residues—at a final concentration 0.8 mM. Nucleic acids were stained with the DNA selective dye Hoechst 33258 (Sigma Aldrich, St. Louis, MO, USA) and viewed in the blue channel (excitation at 351 and 364 nm and emission at 400-480 nm). Autofluorescence was used for imaging photosynthetic pigments at 543 and 633 nm excitation wavelengths (He-Ne) and in the 590-to 800 nm emission range (red channel). Phycoerythrin and chlorophyll *a* fluorescence were detected between 555–595 nm and 690–750 nm respectively by excitation with a 488 nm argon laser. The reflection image (grey channel, excitation at 488 nm and emission at 480 to 490 nm) was used to

visualise the external surfaces and mineral particles. Fluorescence emission was then sequentially collected in the green and red regions of the spectrum. Line averaging (x4) was used to capture images with low noise. Data consists of a set of two dimensional (2D), cross-sectional image in the x-y plane that is captured along the z-axis. Natural fluorescence of chlorophyll pigments and phycobiliproteins was used to trace the internal distribution of microalgae and cyanobacteria. The CLSM data were visualised in different projections (Roldán et al. 2004a) using Imaris software (Bitplane AG Zürich, Switzerland). The side views of the 3D reconstructed images were used to determine biofilm architecture.

RESULTS

The photosynthetic communities that inhabit the cave rocks thrive mainly on the surface. Walls, speleothems, stalagmites and stalactites provide a variety of ecological niches that primarily undergo non-endolithic colonisation. The upper surfaces appeared blue, greenish or grey as a result of photosynthetic growth. The patinas were continuous, arranged mosaically or spotty. Macroscopic views of biofilms from the caves are shown in Fig. 1 a-e.

The microscopic studies revealed the complexity of these communities and their constituent organisms. SEM enabled visualisation of the surfaces (Fig. 2 a-f), whereas CLSM microscopy allowed taxa identification and cell localisation in the intact biofilms. Fluorescence and Con-A were used in tandem to assess cell viability: metabolically active cells are indicated by pigments, which fluoresce red in the cell cytoplasm. Binding of Con-A to polysaccharides outlines the sheaths and cell walls of nearly all microorganisms, including bacteria and actinobacteria. The images revealed traces of inorganic materials such as substrata and calcified sheaths.

Cave of Bats, Zuheros.

Sampling point Z1 was covered by a dark green mucilaginous layer (Fig. 1 a). The microflora present comprised the cyanobacteria *Aphanocapsa parietina*, *Cyanosarcina* cf. *parthenonensis, Gloeocapsa* spp, *Gloeocapsopsis magma, Leptolyngbya* spp., *Nostoc* sp. and *Tolypothrix* sp., the clorophytes *Chlorella* sp., *Desmococcus* sp., *Klebsormidium flaccidum*, *Trebouxia* sp., diatoms and mosses in different states. The cyanobacteria exhibited colourless sheaths. The number of taxa decreased from Z1, at the mouth of the cave and lit by natural light, to Z3, further inside the cave. Indeed, by point Z3, the only species remaining were *Cyanosarcina* cf. *parthenonensis, Chlorella* sp., *Klebsormidium flaccidum* and the diatoms *Diadesmis* sp. and *Nitzschia* sp. Bacteria and actinobacteria (Fig.2a) were widespread and entangled with the photosynthetic organisms. The biofilms that encompassed several taxa generally exhibited stratification, with a continuous upper layer of chlorophytes and a discontinuous bottom layer of cyanobacteria (Fig. 3; Type 1 biofilm). The thickness of their photosynthetic layers was 25.5 ± 4.5 µm. Any diatoms present were located on the surface. In some cases this layer appeared strongly labelled with con-A. This can be attributed to the EPS, which, despite holding the colonies together, also appeared to delineate the different organisms, especially cyanobacteria or green algae in thick coverings.

The biofilms from Z2 had the cyanobacteria *Gloeothece* sp. and *Cyanosarcina* cf. *parthenonensis* at

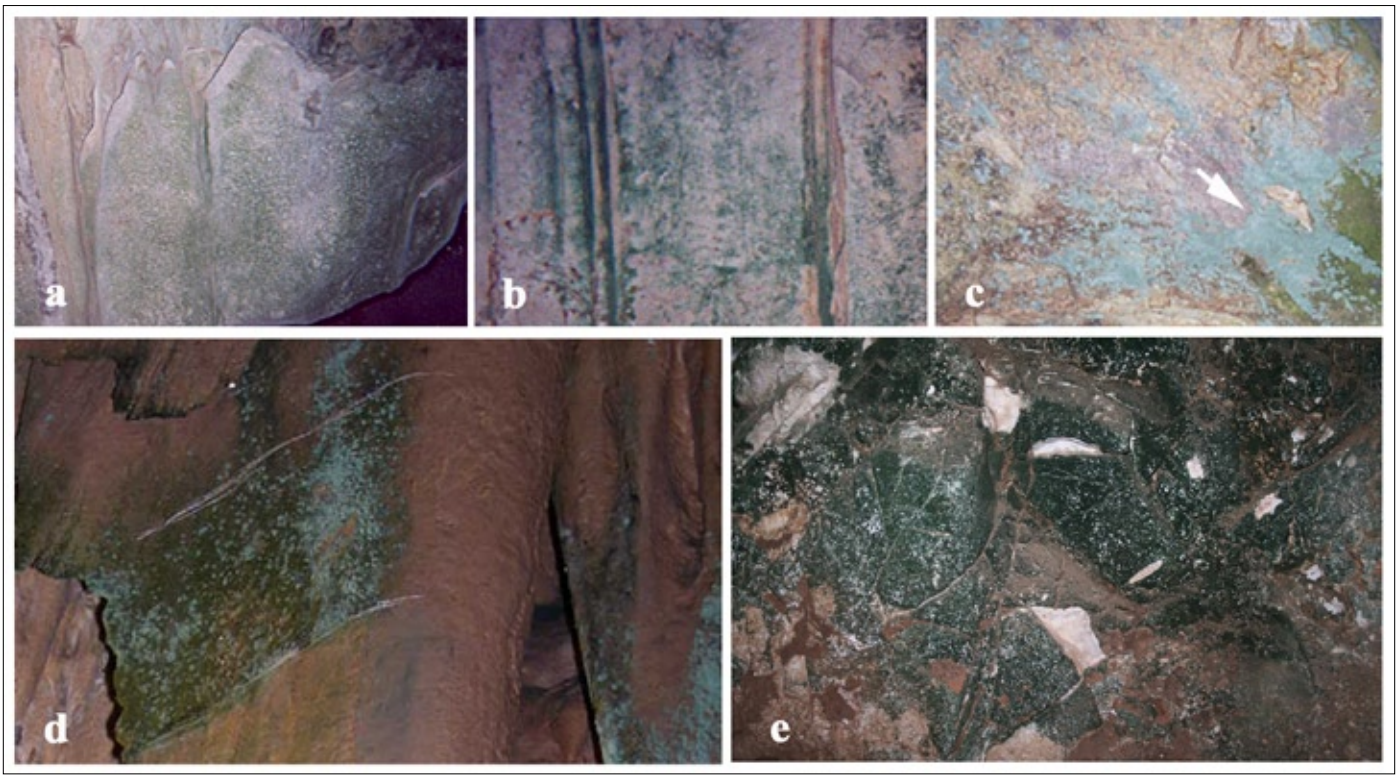

Fig. 1. Macroscopic images from the sampling zones: a. Zuheros (sample Z1); b. Zuheros (sample Z4); c. Zuheros (sample Z7) Leprose lichen (white arrow); d. Collbató (Virgin Cave); e. Nerja (sample N5).

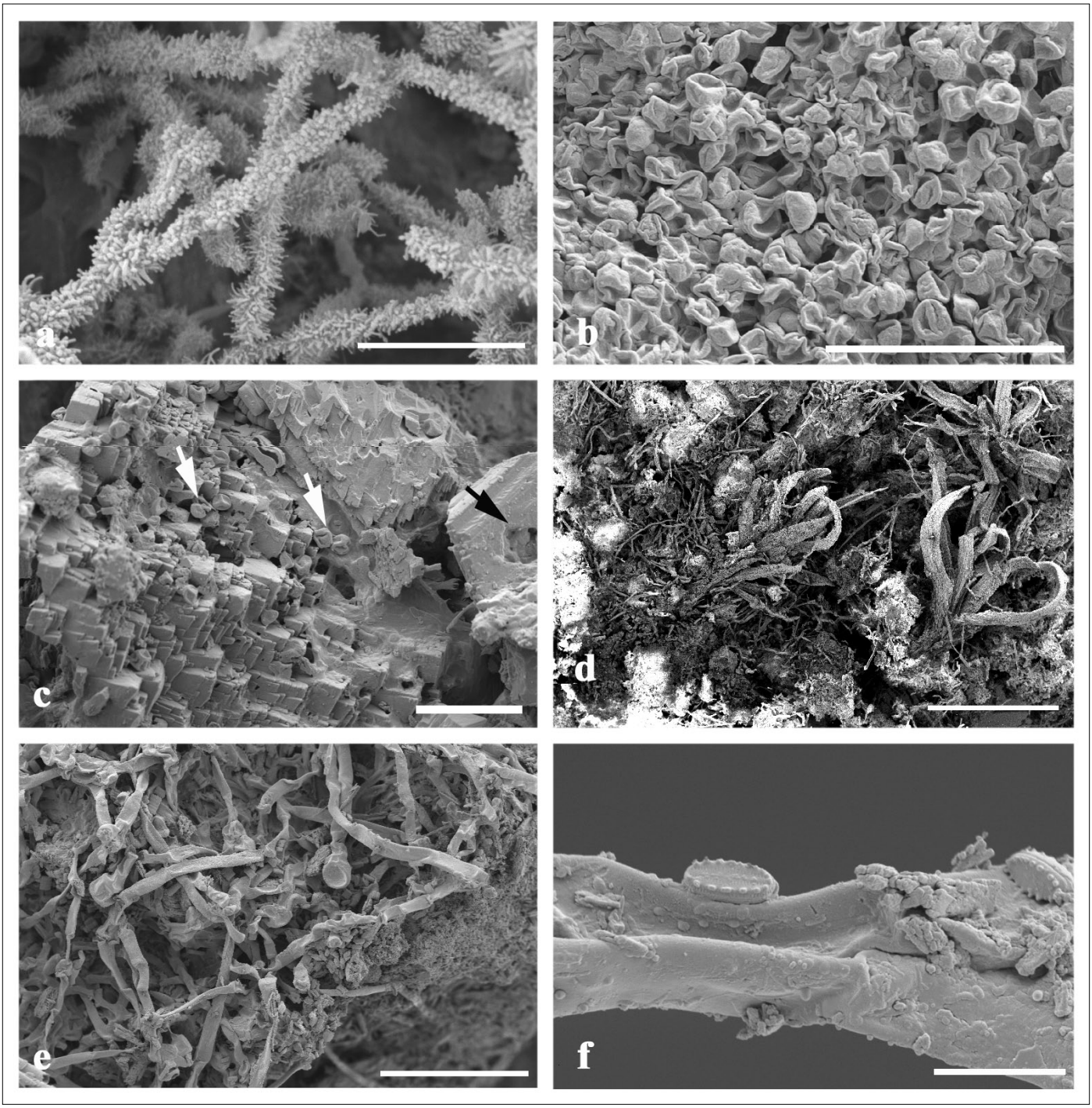

Fig. 2. Scanning electron photomicrographs. a. Z1. Image of a filamentous actinobacterium with crystal formation (scale: 5µm). b. Z4. Rod-shaped *Muriella* sp. (Chlorophyta) forming a continuous layer on the surface of a dolomitic rock. Cells are collapsed due to dessication induced by processing of the samples for SEM (scale: 20 µm). c. Z4. Biofilm formed by a single-celled *chlorophyta* irregularly distributed on a dolomite surface. The white arrows indicate cells on the surface and black arrow shows cells inhabiting a fissure (scale: 20 µm). d. Z6. Biofilm formed of mosses or protonemata epiphyted by *Bacillariophyta* (*Diadesmis* sp.) on a speleothem (scale: 1 mm). e. Z7. Leprarioid lichen *Botryolepraria lesdainii* (scale: 100 µm). f. Z7. High magnification showing *Diadesmis gallica* (diatom) on the lichen *Botryolepraria lesdainii* (scale: 10 µm).

the bottom, on the surface or in holes, and contained *Chlorella* sp., and occasionally scarce diatoms, at the top. In samples from Z2, the thickness of the photosynthetic layers was 13.6 ± 2.2 µm (Type 2 biofilm), whereas in samples from Z3, the predominant biofilm was 12.7 ± 3.6 µm (Type 3 biofilm), which did not present stratification.

Sampling point Z4 (Fig. 1 b) which receives scarce natural and artifical light, showed thin and compact patches of phototrophic growth, following temporary water tracks; their colour varied from bright blue-green to olive green. The photosynthetic layers were either thin, continuous and compact (Fig. 2 b) or irregularly covering the surface (Fig. 2 c). The most frequently identified organisms were *Chlorella* sp., *Chlorosarcinopsis* sp., *Muriella* sp. and *Myrmecia bisecta*. Their microflora also included *Cyanosarcina* cf. *parthenonensis, Leptolyngbya* cf. *lurida, Leptolyngbya* spp., *Phormidium* cf. *tenue, Phormidium* spp., *Chlorella* sp., *Chlorosarcinopsis* sp., *Klebsormidium flaccidum, Muriella* cf. *terrestris, Diadesmis* spp., and *Orthoseira roseana.* Biofilms that only contained one

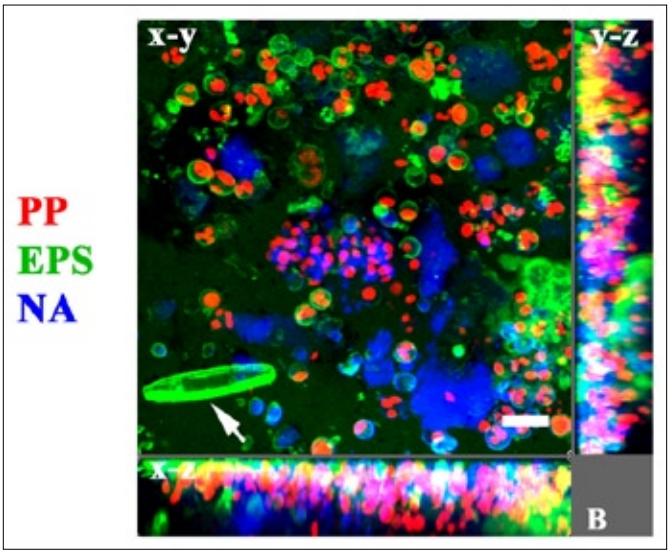

Fig. 3. Confocal photomicrograph. Structure and composition of a stratified Type 1 biofilm, in Zuheros (Z1). Central frame shows the maximum intensity projection of the mixed biofilm. Stratification of the various groups of phototrophic organisms with an upper layer of Chlorophyta (*Chlorella* sp.), a discontinuous bottom layer of cyanobacteria (*Gloeothece* sp. and *Cyanosarcina* cf. *parthenonensis*), and occasionally, scarce diatoms at the top (white arrow) can be seen in the lateral frames. Three-dimensional extended projections in x-y, x-z and y-z views of 42 sections (step = 0.4 µm) in the z-direction of the biofilm. 16.40 µm total thickness.
Colour key: red = autofluorescence of pigments (PP); green = EPS labelled Con-A (EPS), blue = nucleic acids (NA). B = bottom of the sample (scale: 10 µm).

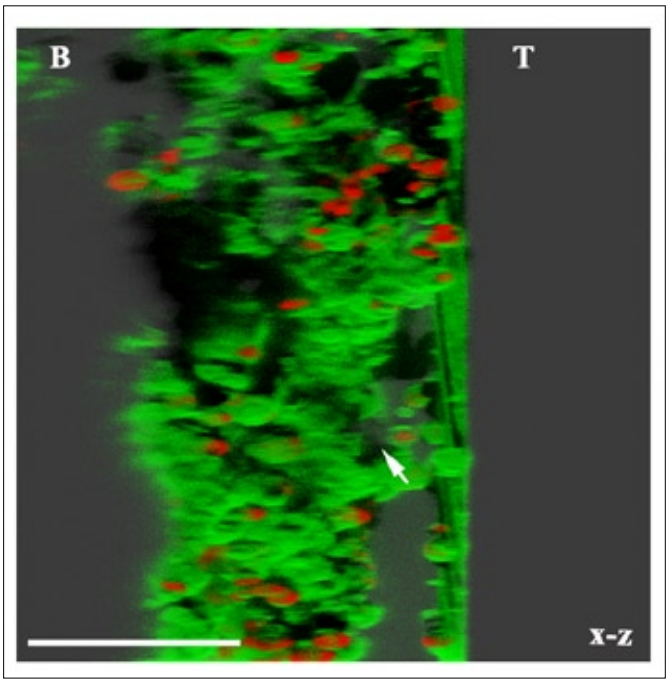

Fig. 4. Confocal photomicrograph. Saggital projection of an unstructured Type 3 biofilm in Zuheros (Z4). a. The distribution of the microorganisms along the x-y axis was irregular; along the x-z axis the biofilm moulded the substrata. Some *Chlorella* sp. were hidden in small crevices. The EPS was distributed heterogeneously. Bubble-like structures or pores were observed (arrow). Colour key: red = autofluorescence of pigments; green = EPS labelled Con-A. T = top of the sample; B = bottom of the sample (scale: 100 µm).

or a few species were flat. Their photosynthetic layer had a thickness of 15.7 µm ± 6.9 µm (Fig. 4). The organisms were irregularly distributed, some patches contained *Cyanosarcina* cf. *parthenonensis* coexisting with *Chlorella* or other chlorophytes (Fig. 5 a-c; Type 2 biofilm). All of these were either on the substrate or hidden inside of it. Their fluorescent layers had a thickness of 16.3 ± 3.8 µm.

At Z6, which only receives irregular artificial light, there was a thin, green layer with a thickness of 14.9 ± 3.2 µm, formed of *Chlorella* sp., either alone or mixed with moss protonema or developed mosses (Fig. 2 d; Type 4 biofilm). In the biofilms dominated by well-developed mosses, the fronds appeared epiphyted by *Diadesmis gallica.* They were only locally abundant on walls of calcite microgours (humid areas with dripping water).

The sampling points near the exit, Z7 and Z8 (Fig. 2 e, f), were covered by a discontinuous layer with irregular colouration. At the outermost points, the stains were greyish and primarily formed of the lichen *Botryolepraria lesdainii*, with the basal layer composed of their phycobiont *Coccobotrys verrucariae*, in free-living state (Fig. 6, Type 5 biofilm), as well as other green algae such as *Trebouxia* sp., which also formed lichens (Fig. 6 b). Some of the lichens appeared to be covered by diatoms (Fig. 2 f) or other microorganisms. At the rock entrances and in the most-protected zones, the patches were greenish-grey, or green and gelatinous when hydrated, owing to the presence of the green algae *Trebouxia* sp., *Chlorella*-like, *Stichococcus bacillaris* and *Leptosira* sp. *Trentepohlia* sp. was present in scarce amounts and did not contribute to the formation of lichens. The different biofilms on the rock varied widely in their thickness. The biofilms composed of diverse chlorophytes had a thickness of 13.7 ± 1.1 µm (Type 3 biofilm), almost the same as that of the biofilms comprised of only a single species (6.5 ± 3.2 µm). Naturally or artificially illuminated crevices under high relative humidity were dominated by mosses, on which grew the centric diatom *Orthoseira roeseana* (Fig. 6 c) and the pennate *Diadesmis gallica*. However, both diatoms were often found dead or in a senescent state, as indicated by their total or nearly total lack of fluorescence.

Biofilms in Nerja.

The patina that colonised the sampling point N5 (Fig. 1 e) grew as a thin, discontinuous layer on surface areas, natural cracks and fissures (Fig. 7 a, b). The dominant photosynthetic organism was *Muriella* sp., located on the surface or in open crevices or pits (Fig. 7 a, b). The photosynthetic layer that covered the rock was very thin, sometimes comprising only a single layer of cells (Type 3 biofilm), although it appeared thicker since this layer covered sharp rock and cracks. *Nostoc* sp. and coccal cyanobacteria were present at very close points (N6) exposed to natural light (Fig. 7 c).

Biofilm in Collbató.

The stalactite surface (Fig. 1 d) was covered with a greyish-white, porous mass primarily composed of the filamentous cyanobacterium *Scytonema julianum*,

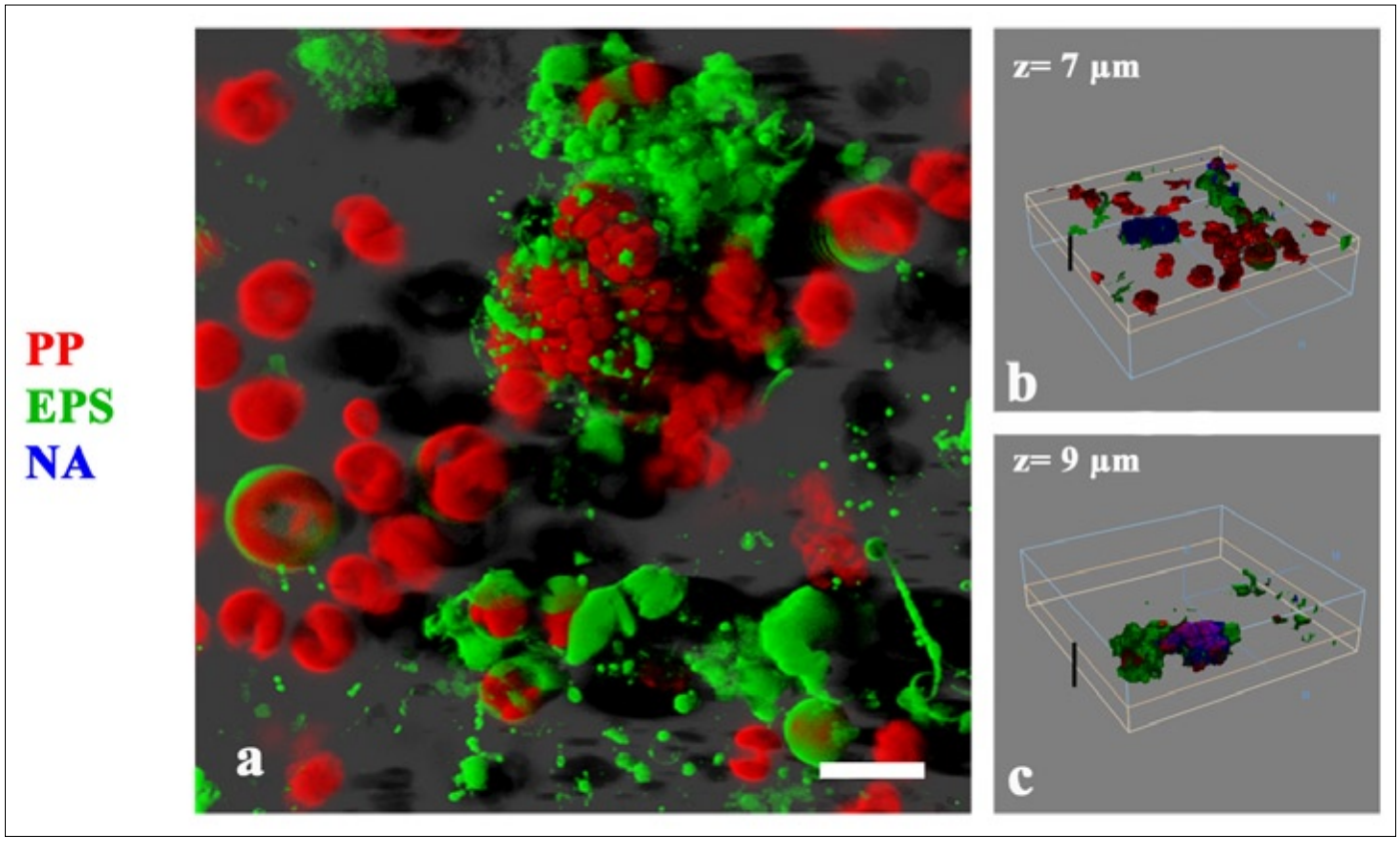

Fig. 5. Confocal photomicrographs. Structure and composition of a stratified Type 2 biofilm in Zuheros (Z4). a. Two-channel image created by the Simulated Fluorescence Projection (SFP) method of 69 x-y optical sections (z step = 0.3 µm). Thickness = 20.40 µm. b. c. 3D perspective projection in the ray-tracing method of Fig. 5a. Selected regions from top and bottom levels; each level has a particular microbial composition and mucilage abundance. b. The surface level of the biofilm (Z = 7 µm) is mainly composed of *Myrmecia bisecta* (Chlorophyta). c. The bottom level (Z = 9 µm) is composed of *Cyanosarcina* cf. *parthenonensis* (Chroococcales) which is sometimes located in deeper parts of the substratum (chasmoendolithic growth) (scale: 10 µm). Colour key: red = autofluorescence of pigments (PP); green = EPS labelled Con-A (EPS); nucleic acids (NA).

which exhibited a thin calcified external sheath. This species was entangled with *Leptolyngbya* spp., *Klebsormidium flaccidum* and moss protonema. The diatom *Diadesmis contenta* and the green algae *Chlorella* sp., *Coccobotrys verrucariae* and *Myrmecia biatorellae* were located primarily at the top and were irregularly distributed. Spots of *Nostoc punctiforme* and *Gloeocapsopsis magma* were located deep within the substrata (chasmoendolithic growth). The structure of the biofilm was due to *Scytonema julianum*, (Fig. 7 d, Type 6 biofilm) which was sometimes accompanied by other filamentous cyanobacteria or mosses. Any diatom present was located in the upper layer.

All these microscopy observations revealed that the community of microorganisms were organised in biofilms characterised by spatial structure. The patinas appeared to be distributed spatially and were very heterogeneous in thickness, density and organism composition. The biofilms were compact and thin, from 6.45 to 25.47 µm thick in Zuheros, although they appeared thicker when moulding the substrata irregularities (from 50.31 to 167 µm). This indicates a very rough substratum, which was even more irregular in Nerja, in which the layer of photosynthetic organisms traced the highly irregular surfaces. As a rule, the biofilms were less than 1 mm thick, except in humid areas close to lamps, where mosses contributed to the structure. EPS were irregularly distributed in the biofilms and were more abundant in the upper layers. The cyanobacteria were on top of the substrata or were hidden in it, whereas the green algae were always found on the surface, whether growing directly on the substrate or on top of the other organisms in the biofilm. No endolithic growth was observed. Schematics of Types 1 to 6 biofilms are illustrated in Fig. 8 a-f.

DISCUSSION

The examples described above are all cave habitats; from an anthropocentric perspective, this translates to hypogean and dark. Hence, their primary common stress factor is light shortage, followed by humidity, a lack of nutrients, and to a far lesser extent, temperature (Smith & Olson, 2007). The amount of light varies with cave type. It also varies within caves according to gradients from the mouth to the interior.

Areas near cave entrances are generally affected by environmental changes and show major fluctuations (Hoffmann, 2002; Roldán et al., 2004b; Vinogradova et al., 1998). In the spring and at the end of the summer, the afternoon light remains intense, coinciding with maximum temperature and minimum relative humidity. Temperature, relative humidity and light show a clear gradient from the entrance up to a certain depth of the interior of the cave, beyond which

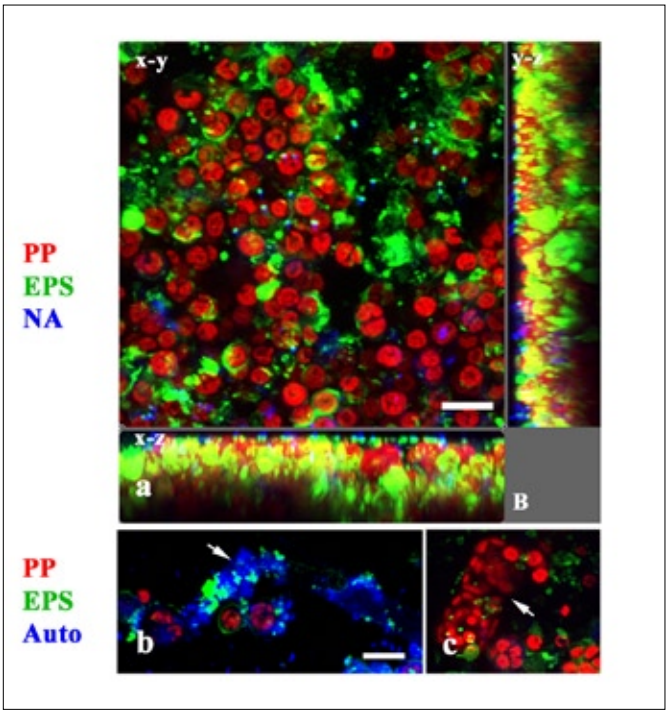

Fig. 6. Confocal photomicrographs. Structure and composition of a Type 5 biofilm in Zuheros (Z7). Sampling points near the exit, with natural light. a. Biofilm formed by an upper layer of the leprose lichen *Botryolepraria lesdainii*, with or without diatoms, and a bottom layer of *Coccobotrys verrucariae* (Chlorophyta), either lichenised or free-living (B = bottom of the sample). Three-dimensional extended projection in x-y, x-z and y-z views of 90 optical sections (z step = 0.2 µm) in the z-direction of the biofilm. Thickness = 17.80 µm (scale: 10 µm). b. Some lichens presented natural blue fluorescence (white arrow). c. Associated organisms were other green algae such as *Trebouxia* sp. and diatoms (e.g. *Orthoseira roeseana*, white arrow) (scale: 10 µm). Colour key: red = autofluorescence of photosynthetic pigments (PP); green = EPS labelled Con-A (EPS); blue = nucleic acids (NA) or autofluorescence of lichens (Auto).

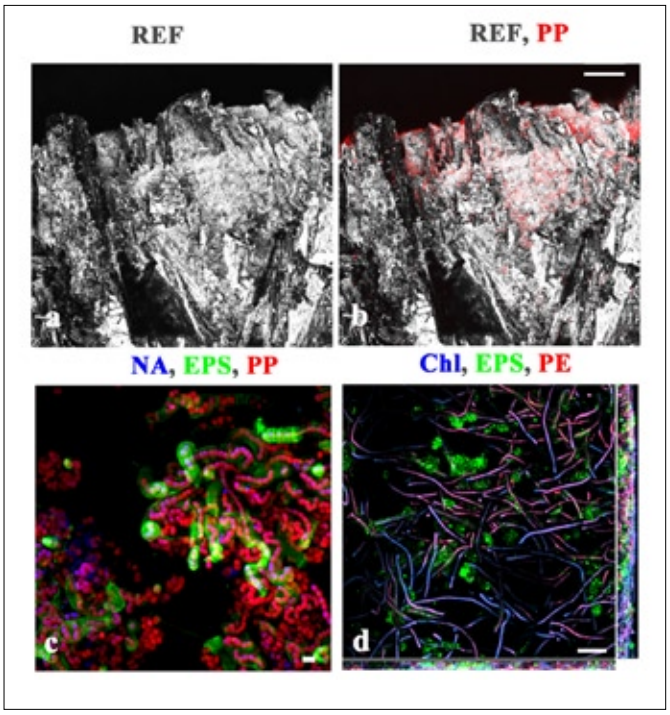

Fig. 7. Confocal photomicrographs. a. Reflection projection showing inorganic calcareous material from Nerja (N5). b. Reflection, as in Fig. 7, a, plus spatial distribution of pigment autofluorescence. Single celled chlorophytes are distributed in a thin layer penetrating the fissures and fractures (chasmoendolithic growth) (scale: 100 µm). c. Maximum intensity projection from a biofilm composed of *Nostoc* sp. (N6), showing colonies with and without EPS production (scale: 10 µm). d. Structure and composition of a Type 6 biofilm in Collbató (Virgin Cave) formed mainly of *Scytonema julianum*. Chlorophyll-a and phycoeritrin autofluorescences can be distinguished in different emission wavelengths (scale: 100 µm). Colour key: white = reflection (REF); red = autofluorescence of photosynthetic pigments (PP) or phycoerithrin (PE); green = EPS labelled Con-A (EPS), blue = nucleic acids (NA) or autofluorescence of chlorophyll *a* (Chl).

they remain stable. Deep within the cave, relative humidity is usually high (Pentecost & Whitton, 2000); there is little or no variation during the diurnal cycle, and only slight differences were observed between the measurements from rainy and arid seasons, and between those from winter and summer. From the mouth of the cave to the furthest sampling point inside, the organisms were organised into mosaics or belts according to environmental conditions. The diversity of microalgal and cyanobacterial species decreased with decreasing light (Roldán et al., 2004b).

Approximately 350 taxa of photoautotrophic microflora have been reported for hypogean environments, although some of these were identified in culture and are unable to survive in dim environments (Hoffmann, 2002). The caves whose literature was surveyed for this work either exhibited a much lower total number of species (Asencio & Aboal, 2000; Friedmann, 1964; Vinogradova et. al., 1998; Roldán et al., 2004b), or only their cyanobacteria were studied, which nevertheless represent more than half of the total taxa and tend to dominate with associations similar to those described by Golubic (1967) and those cited in the review by Pentecost & Whitton (2000). Coccoid forms are more abundant in illuminated areas and dripping sites. Filamentous forms tend to be more diverse in darker or more humid locations (Vinogradova et al., 1998). Exceptions include *Scytonema julianum*, which can bear strong fluctuations (Leclerc et al., 1983; Coute & Bury, 1988): it thrives in sheltered walls that receive light peaks of 1800 µE $m^{-2}s^{-1}$, yet it grows in culture down to 21 µE $m^{-2}s^{-1}$ (Ariño et al., 1997) and can grow in caves with relatively low humidity (down to ca. 50%) (Aboal et al., 1994).

In contrast, other filamentous cyanobacteria such as *Geitleria calcarea*, *Herpyzonema pulverulentum* and *Loriella* sp. require long term stability and humidity near the dew point and grow slowly in levels of light down to 1 µE $m^{-2}s^{-1}$ (Ariño et al., 1997, Leclerc et al., 1983, Roldán et al., 2004b); none of these were found during this study.

For environments in which the only source of light is artificial, the number of species is even lower; this is true for caves (Smith & Olson, 2007) as well as similar hypogean environments (Albertano et al., 1994, 2003), in which mosses and green algae are more abundant near the lamps, provided that the ambient humidity is near the saturation point or there is dripping. In these artificial habitats, in which

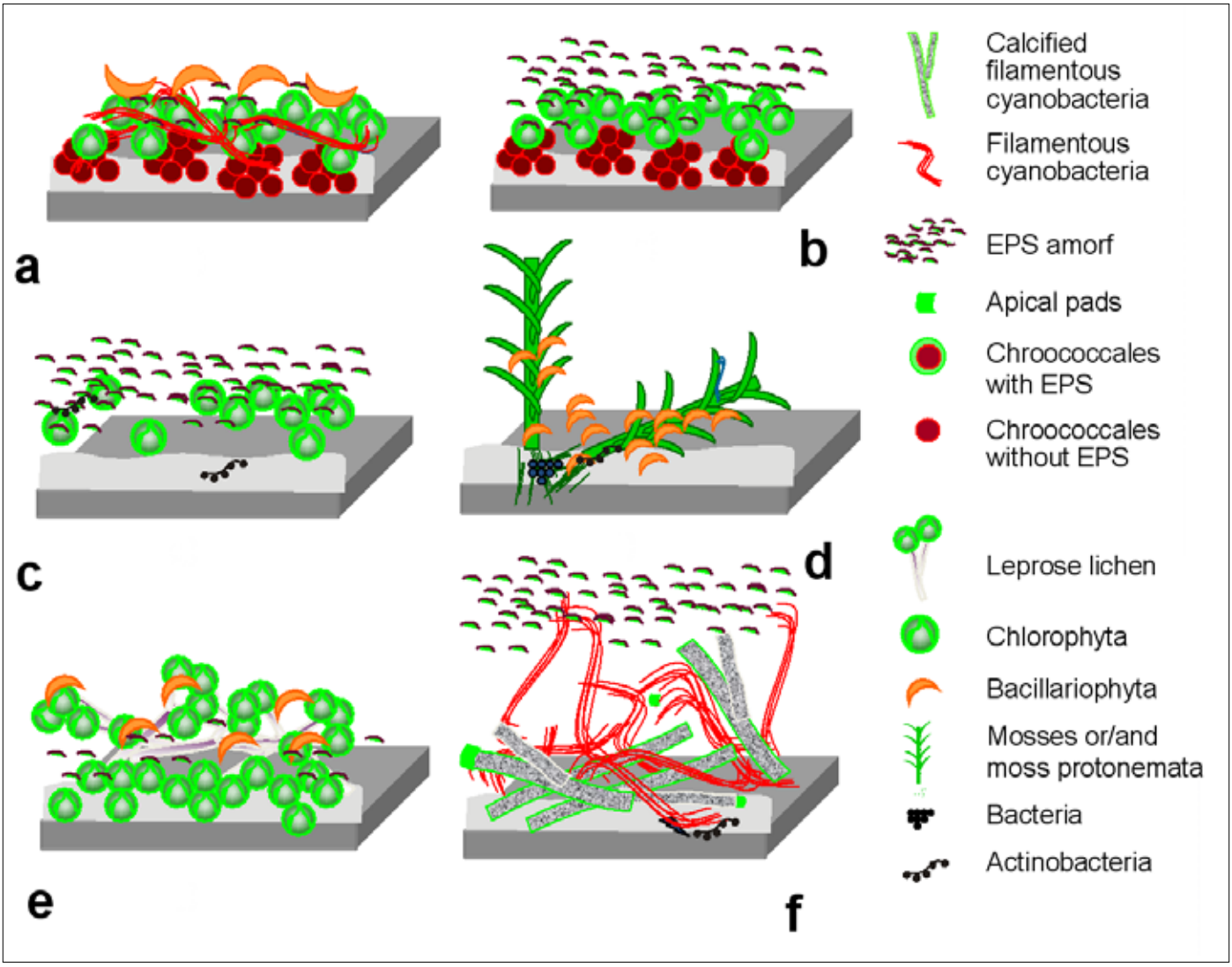

Fig. 8. Conceptual drawings of biofilm structure (Types 1-6) from different caves on various substrata, according to species dominance and depth distribution inside the biofilm. a. Type 1 biofilm: encompassed several taxa and generally exhibited stratification, with a continuous upper layer of chlorophytes and diatoms and a discontinuous bottom layer of cyanobacteria. b. Type 2 biofilm: formed by very few photosynthetic organisms, but still stratified. c. Type 3 biofilm: formed by one or very few organisms, without stratification. d. Type 4 biofilm: Formed by mosses or moss protonemata plus other photosynthetic organisms. e. Type 5 biofilm: formed by lichens plus other photosynthetic organisms. f. Type 6 biofilm: structured by calcified cyanobacteria in addition to other photosynthetic organisms.

light is not continuous, the additional stress factor which appears to compromise the diversity of taxa is stability. The most affected species are cyanobacteria, which can not grow fast enough in these transient habitats. Diatoms are also affected, although their quantification is not easy, owing to their resistant valves, which remain even when they are dead. Measurements by CLSM—which enables separate observation of live diatoms (which fluoresce) from dead ones (which do not fluoresce)—can be used as an indicator of activity, and consequently, as a tool to determine the health of populations. Hence, an increase in levels of scavengers or actinobacteria at certain points suggests population substitution as a result of variable conditions.

Photosynthetic microorganisms exhibit considerable interspecific variation in their ability to persist under prolonged darkness (Albertano et al., 1991). To withstand extended periods of light deprivation, photosynthetic cells must maintain viability. The conditions of light deprivation and burial have been widely studied in lakes and rivers. The same taxa capable of tolerating conditions of low light are also likely to exhibit high resistance to prolonged light deprivation (Peterson, 1996). Another factor that can favour long-term survival in low- or zero-light conditions is heterotrophy. Although most microalgae and cyanobacteria grow photoautotrophically, some can grow heterotrophically. Owing to Chodat's (1909) pioneering work on the culture of microscopic algae, including *Chlorella* and *Coccomixa*, the capacity to which these organisms can grown on organic substrata is known: they grow heterotrophically via an active glucose uptake system. This process has been described for phototrophic cells (Chen & Chen, 2006), including *Plectonema boryanum* (Raboy & Padan, 1978), which has been broadly described in caves.

The aforementioned differences among the organisms and in the physicochemical characteristics of the environment could explain the species assemblies and microscale structural organisation. The biofilms described from the entry (Types 1- 3 biofilms) and the

exit (Type 5 biofilm) of Zuheros are characteristic of the mouths of caves (Hernández-Mariné et al., 2001b; Ariño et al., 1997). A gradient was observed in which the diversity and thickness of biofilms decreased with decreasing light. Type 1 biofilm was marked by vertical stratification, apparently following the vertical gradient of light, whereby diatoms and green algae are located on the surface, and cyanobacteria are located at the bottom. In contrast, Type 3 biofilm was unstructured and comprised of one to a few taxa; dominant chlorophytes that they contain can be considered taxa characteristic of early colonisation. The green colour of these biofilms, as well as the lack of colour in areas adjacent to those in which they grow, which are exposed to the same light, may be indicative of a lack of water during dry periods. Type 3 biofilm found on highly irregular substrates (*e.g.* those of Nerja N5) were observed to grow deep into the interior of rocks up to the point at which light was insufficient, exhibiting chasmoendolithic growth. Mosses only dominated at dripping points close to light sources. On top of the mosses were found other organisms, such as diatoms or filamentous cyanobacteria (Albertano et al., 1994). Type 6 biofilm was heterogeneous in structure and primarily composed of *Scytonema julianum*.

The three-dimensional structures of biofilms from many other extreme environments have been described. This has required that the techniques for sampling and observation do not compromise the architecture itself. Examples can be found for hot and cold deserts, (Wynn-Williams, 2000 and references therein), desert soil crusts (Garcia-Pichel & Belnap, 1996; Nienow & Friedmann, 1993), endolithic environments (Walker & Pace 2007), and extremely acidic environments (Aguilera et al., 2007), in which micrometre scale stratification can be observed. Vertical zonation is especially visible in the benthic microbial mat communities of Antarctica (Vincent, 2000), in which blue-green coloured strata, usually located towards the bottom of the mats, have been described. This heterogeneous distribution reflects different adaptation strategies used by microorganisms, and should provide certain advantages, at least for some of the constituent microorganisms in the structure. Understanding the factors that control the microorganisms as well as the effects of environmental stress on biofilm formation in dim habitats will require further studies employing better methods than those currently available.

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